Highly efficient neutralization of HIV with recombinant CD4-immunoglobulin molecules

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THE human immunodeficiency virus type 1 (HIV-1) exploits the cell surface CD4 molecule to initiate the infection[1–4] which can lead, eventually, to acquired immunodeficiency syndrome (AIDS). The HIV-1 envelope protein, gp120, interacts specifically with CD4 and soluble CD4 molecules have been shown to inhibit HIV infectivity *in vitro*[5–9]. Effective inhibition *in vivo* may, however, require more potent reagents. We describe here the generation of molecules which combine the specificity of CD4 and the effector functions of different immunoglobulin subclasses. Replacing the VH and CH1 domains of either mouse γ2a or μ heavy chains with the first two N-terminal domains of CD4 results in molecules that are secreted in the absence of any immunoglobulin light chains. We find that the pentameric CD4–IgM chimaera is at least 1,000-fold more active than its dimeric CD4–IgG counterpart in syncytium inhibition assays and that effector functions, such as the binding of Fc receptors and the first component of the complement cascade (Clq), are retained. Similar chimaeric molecules, combining CD4 with human IgG were recently described by Capon *et al.*[10], but these included the CH1 domain and did not bind Clq. Deletion of the CH1 domain may allow the association and secretion of heavy chains in the absence of light chains[11], and we suggest that the basic design of our constructs may be generally and usefully applied.

We have previously produced chimaeric proteins consisting of different domains of CD4 and immunoglobulin constant region of mouse κ (Mκ). If we denote the four suggested extracellular domains of CD4 protein starting at N-terminus by 1, 2, 3 and 4 (ref. 12), the following proteins were generated: 1·Cκ, 1·2·Cκ, 1·2·3·Cκ and 1·2·3·4·Cκ (ref. 13). All these molecules retained their gp120 binding activity, suggesting that the first Vκ-like domain is sufficient. We first attempted to produce secreted CD4-immunoglobulin chimaeras with specificity against gp120 by generating double transfectants producing 1·Cκ and normal immunoglobulin heavy chains. This approach failed, however, and we could not detect assembly into complete molecules, even though a number of heavy chains with different variable regions were tested.

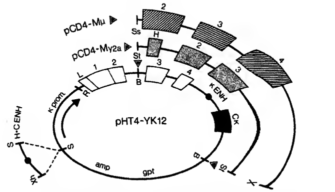

FIG. 1 Immunoglobulin-CD4 chimaeric constructs. The plasmid pHT4.YK12 encodes all four extracellular domains of CD4 linked to the mouse Igκ constant regions. CD4–Mμ and pCD4–Mγ2a encode the first two N-terminal CD4 domains linked to mouse Igμ (CH2, CH3, CH4) and mouse Ig γ2a (Hinge, C_H2, C_H3) constant regions, respectively. Numbers above the boxes refer to exons coding for different domains. ENH=Enhancer, L=Leader, H=Hinge, B=*Bam* HI, R=*Eco* RI, S=*Sal* I, Ss=*Sst*, St=*Stu* I, X=*Xba* and Xh=*Xho* I. METHODS. The CD4-κ expression vector pHT4-Y1 (ref. 9) was modified by replacing the immunoglobulin heavy chain promoter by a more efficient immunoglobulin κ promoter derived from the vector pKm 1 (ref. 32). The κ promoter was first subcloned into an intermediate vector PUC 18 as a *Bgl* II/*Sal* I fragment, then transferred as a 2.2 kilobase (kb) fragment [*Hind* III(blunt)/*Eco* RI] into pHT4-γi [*Xba* I(blunt)/*Eco* RI], to generate pHT4-YK12. The *Bam* HI fragment of pHT4-YK12 (encoding domains 3 and 4 of CD4 and Cκ) was then replaced by either a 3.5 kb *Sdt*/*Xba* I fragment containing C_H2, C_H3 and C_H4 constant region exons of mouse Igμ or a 3.0 kb *Stu* I fragment encoding the hinge, C_H2 and C_H3 constant region exons of mouse Igγ2a. All fragments were blunt-ended before ligation into the vector. The constructs pCD4–Mμ and pCD4–Mγ2a were completed by inserting a heavy chain enhancer as a *Sal* I/*Xho* I fragment into the unique *Sal* I site 5′ of the promoter.

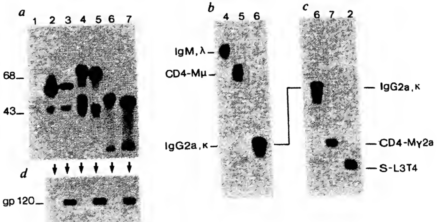

FIG. 2 Characterization of the chimaeric molecules. Stable transformants were obtained by transfecting CD4 constructs into X63-0 myeloma cells by protoplast fusion. *a*–*c*, Western blot analysis of immunoprecipitated chimaeric or natural immunoglobulin molecules from cell culture supernatants after 10% PAGE run under reducing conditions (*a*) or after 3–7% gradient PAGE (*b*) or 7% PAGE (*c*) run under non-reducing conditions. Non-transfected X63-0 (1), soluble murine CD4, L3T4 (ref. 9) (2), HT4-YK12 (3), B1.8 (IgM, λ) (4), CD4–Mμ (5), UPC10 (IgG2a, κ) (6), CD4–Mγ2a (7). Samples 1–3 were immunoprecipitated with rabbit anti-mouse κ, samples 4 and 5 with rabbit anti-mouse IgM, samples 6 and 7 with protein A. All antibodies and protein A were immobilized on Sepharose 4B. The results were visualized with a mixture of rabbit anti-mouse Ig κ, μ and γ antibodies in a first step, followed by radioactive donkey-anti-rabbit immunoglobulin. Standard molecular weight markers are shown on the left (in thousands). The background bands in a (1–5), of relative molecular mass ~45,000 are due to rabbit IgG released from the Sepharose when the samples were prepared for electrophoresis. *d*, Co-immunoprecipitation under identical conditions as those in *a*–*c*, but in the presence of metabolically labelled gp120 from the supernatant of HIV-1-infected H9 cells, was followed by PAGE and fluorography.
METHODS. Four million H9/HTLV-III cells[33] were pulse-labelled as previously described[34] with [^{35}S]cysteine for 4 h. The cells were then transferred into 4 ml of fresh medium and incubated for a further 14 h. After the chase the cells were quickly removed by pelleting at 400*g* and the supernatant was subjected to 150,000*g* sedimentation for 1 h. Cleared supernatant (50 μl) was then incubated with the indicated culture fluids for 14 h at 4 °C and the immunoprecipitations were performed as described above.

EXHIBIT K

The successful approach relied on two observations. First, 1·2·Cκ and 1·2·3·4·Cκ were secreted, whereas 1·Cκ and 1·2·3·Cκ were not, suggesting to us that CD4 domains associate pairwise into two stable units consisting of 1·2 and 3·4 domains respectively. Second, the analysis of human heavy-chain disease proteins has shown that heavy chains of different subclasses can be secreted without light chains when the first constant region domain is deleted[11]. We therefore constructed plasmids in which the exons encoding the first two N-terminal domains of CD4 were linked to all but the V_H1 and C_H1 domains of either the mouse μ heavy chain (CD4–Mμ) or the mouse γ2a heavy chain (CD4–Mγ2a) (see Fig. 1).

Both CD4–Mμ and CD4–Mγ2a were found in the culture supernatants at levels of 1–5 μg ml^{-1} after the corresponding constructs were introduced into a myeloma cell line X63-0 (Fig. 2). Immunoprecipitations of the secreted proteins with relevant antibodies or protein A and subsequent western blot analyses showed that the molecules produced had the expected apparent molecular weights (Fig. 2). A similar analysis under non-reducing conditions indicated that CD4–Mμ was most probably secreted as a pentamer, whereas CD4–Mγ2a formed dimers, consistent with the fact that CD4–Mγ2a bound to protein A (ref. 14) (Fig. 2*b*, *c*). Both CD4–Mμ and CD4–Mγ2a, as well as the previously produced CD4–Mκ, were able to bind to HIV gp120 (Fig. 2*d*). Because recombinant gp120 was not available, we used metabolically labelled preparations of gp120 derived from the supernatants of HIV-infected (H9) cell cultures in our co-immunoprecipitation tests.

Importantly, the effector functions of normal immunoglobulin molecules, such as binding to Fcγ receptors and Clq were kept intact in the hybrid molecules (Fig. 3). This suggests that removal of the CH1 domain does not create major structural alterations

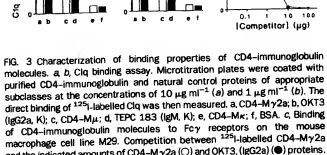

FIG. 3 Characterization of binding properties of CD4–immunoglobulin molecules. *a*, *b*, Clq binding assay. Microtitration plates were coated with purified CD4–immunoglobulin and natural control proteins of appropriate subclasses at the concentrations of 10 μg ml^{-1} (*a*) and 1 μg ml^{-1} (*b*). The direct binding of ^{125}I-labelled Clq was then measured. a, CD4–Mγ2a; b, OKT3 (IgG2a, K); c, CD4–Mμ; d, TEPC 183 (IgM, K); e, CD4–Mκ; f, BSA. *c*, Binding of CD4–immunoglobulin molecules to Fcγ receptors on the mouse macrophage cell line M29. Competition between ^{125}I-labelled CD4–Mγ2a and the indicated amounts of CD4–Mγ2a (○) and OKT3, (IgG2a) (●) proteins. METHODS. *a* and *b*, Polyvinyl chloride microtitration plates were coated with 100 μl of test and control proteins in saline at concentrations of 10 μg ml^{-1} (*a*) and 1 μg ml^{-1} (*b*). After overnight incubation the plates were blocked with 1% BSA solution and binding of ^{125}I-labelled human Clq (~2 ng, 50,000 c.p.m.) was measured after incubation for 6 h at room temperature. Radiolabelling of Clq (a gift from Dr A. Erdei) was carried out by the Iodogen method, according to the recommendations of the manufacturer (PIERCE) *c*, Fcγ receptor binding was assayed by incubating 2×10^6 M29 mouse macrophage cells (a gift from Dr G. Stockinger) with ^{125}I-labelled CD4–Mγ2a (200 ng), plus competitor proteins in 100 μl medium containing 5% fetal calf serum for 1 h at 4 °C. After incubation the samples were centrifuged through a 200 μl cushion of fetal calf serum and the radioactivity in the pellets was measured. The radioactivity obtained after adding 500-fold excess of unlabelled IgG2a (OKT3) was assumed to be due to nonspecific binding and was subtracted before calculating the percentage inhibitions shown. Radiolabelling was performed as described above.

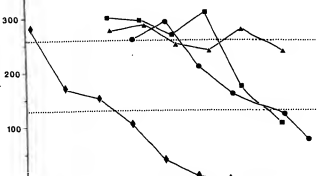

FIG. 4 Inhibition of syncytium formation by different recombinant proteins. The number of syncytia is plotted against the final concentrations of the recombinant proteins (◆) CD4–Mμ; (▩) CD4–Mγ2a; (●) CD4–Mκ; (▲) L3T4–Mκ (ref. 9). The dotted lines indicate the number of syncytia in the absence of any inhibitory proteins and the number at 50% inhibition.
METHODS. A pretitrated amount of HIV-1-HAN was incubated at room temperature with serially diluted recombinant proteins for 30 min. Thereafter, 25 μl of each mixture was transferred in triplicate into the wells of a 96-well plate which contained 25,000 MT-2 cells^{35} per well in 50 μl of medium. After 3 days culture, 100 μl of fresh medium was added per well and after 5 days the syncytia were counted. The sums of syncytia of the triplicates were used for inhibition curves. HIV-1-HAN has been isolated from the PBL of an AIDS patient. Partially determined nucleotide sequence of HIV-1-HAN shows about 90% sequence homology to HTLV-III$_B$ (U. Sauermann and J. Mous, unpublished observation). Culture supernatants of MT-2 or Jurkat cells infected with HIV-1-HAN contained ten times more syncytium-forming capacity than simliar culture supernatants of H9/HTLV-III$_B$ cells.

in the regions of the CH2 domain responsible for Clq and Fcγ receptor binding.[13,16]

To assay the biological activity of these molecules, we tested them in the syncytium inhibition assay[17,18] (Fig. 4). CD4–Mμ was by far the best inhibitor of syncytium formation: 50% inhibition was obtained at a concentration of 10 ng ml^{-1} which was about 1,000-fold less than the concentration (~10 μg ml^{-1}) of CD4–Mγ2a and CD4–Mκ proteins needed for the same effect. Complete abolition of syncytia formation was possible with CD4–Mμ at concentrations of about 1–2 μg ml^{-1}. The CD4–Mκ protein exists as a noncovalently-associated dimer (data not shown), possibly due to the Cκ portion of the molecules. Thus it seems that the effectiveness of these molecules increases as a function of their valence. Truly monovalent CD4, which we do not have, has not been compared directly with the dimeric forms of recombinant CD4 molecules (CD4–Mγ2a and CD4–Mκ) in this particular assay.

Soluble CD4 provides the optimal specificity for neutralization of the HIV-1 for many reasons. First, the strength of the interaction of the CD4 and gp120 is very high, of the order of 10^{-9} M (refs 5, 19). Second, the genetic variants of HIV-1 (refs 20–23) which emerge frequently during the infection must retain their CD4-binding properties to maintain their infectivity. Third, the immunity against gp120 acquired during the infection can have serious deleterious effects on the immune system: free gp120 which is shed from the virus[24,25] can be trapped specifically on the CD4-positive cells and in this way the non-infected cells can become targets of various forms of anti-gp120 immune attacks[26–29]. Passive immunity based on CD4 specificity would avoid this bystander destruction because it would discriminate between gp120 molecules which are already bound on cell-surface CD4 and those molecules which are produced by infected cells.

Although the human CD4–immunoglobulin chimaera reported by Capon *et al.*[10] was secreted, they found that a similar hybrid protein, based on the mouse γ_1 heavy chain, was retained intracellularly. We have also noticed that hybrid molecules containing the C_{H1} domain, for example CD4–IgM chimaeras, are not secreted (unpublished observation) and we suspect that in the absence of immunoglobulin light chains, the hydrophobic face of the C_{H1} domain interacts strongly with the heavy chain binding protein, thus preventing secretion[10,31].

We believe that hybrid proteins which combine the specificity of CD4 with the multivalency and effector functions of different immunoglobulin subclasses could provide a realistic approach to AIDS therapy. We also think that our approach to designing hybrid immunoglobulin molecules could be applied more generally for building novel immunoglobulin molecules. □

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Activation of HIV gene expression during monocyte differentiation by induction of NF-κB

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THE latent period of AIDS is influenced by factors which activate human immunodeficiency virus (HIV) replication in different cell types. Although monocytic cells may provide a reservoir for virus production *in vivo*[1–3], their regulation of HIV transcription has not been defined. We now report that HIV gene expression in the monocyte lineage is regulated by NF-κB, the same transcription factor known to stimulate the HIV enhancer in activated T cells[4]; however, control of NF-κB and HIV in monocytes differs from that observed in T cells. NF-κB-binding activity appears during the transition from promonocyte to monocyte in U937 cells induced to differentiate *in vitro* and is present constitutively in mature monocytes and macrophages. In a chronically infected promonocytic cell, U1, differentiation is associated with HIV-1 replication as well as NF-κB binding activity. These findings suggest that NF-κB binding activity is developmentally regulated in the monocyte lineage, and that it provides one signal for HIV activation in these cells.

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We transfected monocytic cell lines from progressive stages of differentiation with a plasmid containing the HIV enhancer linked to the chloroamphenicol acetyltransferase (CAT) gene. Twenty-four hours after transfection, cells were incubated in medium alone or in the presence of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Expression of the HIV enhancer was induced by TPA in two immature monocyte leukaemia lines, a human granulocyte-macrophage leukaemia, HL-60, and the human promonocytic line, U937 (Fig. 1*a*). TPA treatment did not augment CAT expression in the mature macrophage leukaemic cells, THP-1, P388D1, or PU5-1.8, which showed higher basal activity (Fig. 1*b*; note scale changes).

Using a mutant HIV-CAT plasmid containing alterations in both κB sites[5], we showed that induction of HIV-CAT expression in the immature lines, HL-60 and U937 (Fig. 1*a*), and constitutive expression in the mature lines was dependent on the κB sites (Fig. 1*b*). This suggested that NF-κB is present in the induced progenitors and in the mature cells, and we therefore looked for NF-κB binding activity in nuclear extracts from these cell lines. NF-κB binding activity in the immature lines, HL-60 and U937, was induced by TPA, whereas in the mature macrophage lines, THP-1, PU5-1.8, and P388D1, it was constitutively expressed (Fig. 2*a*). We then determined whether NF-κB binding activity is present in normal human monocytes and/or macrophages. Nuclear extracts were prepared from human peripheral blood monocytes or adherent mononuclear cells, and NF-κB binding activity was found in both cell types as well as in mouse peritoneal macrophages (Fig. 2*b*). NF-κB binding is therefore constitutively active in normal and neoplastic mature mononuclear phagocytes, including blood monocytes and adherent macrophages.

Treatment of immature monocytes with TPA, or the water-soluble phorbol-12, 13-dibutyrate[10] (PDB) (which is more easily removed from cells) causes differentiation into mature monocytes and macrophages, as judged by changes in cell growth, morphology, surface glycoproteins, and phagocytic function (Fig. 3, see also refs 11–15). HL-60 cells treated with PDB acquired characteristics of mature macrophages, displaying growth arrest, increased phagocytosis, adherence, FcR and Mo 1 expression. At the same time, these cells began to express NF-κB binding activity which persisted even two days after